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13. Abstract *(Maximum 200 Words)* Steroid hormones, estrogen and progesterone, and their intracellular receptors play an important role in the development and progression of breast cancer. Coactivator proteins modulate the biological activity of these hormone receptors. We have cloned an E3 ubiquitin-protein ligase enzyme, E6-associated protein (E6-AP) as a coactivator of steroid hormone receptors. The purpose of this research is to explore the possibility that the altered expression of E6-AP may contribute to the development of breast cancer. We propose to explore this by developing animal models for overexpression and loss of function of E6-AP and then relate these observations to the clinical setting by studying the expression patterns of E6-AP in various human breast tumor biopsies. In this progress report, we report that we have successfully generated an E6-AP overexpression model. In order to study the effect of loss of function of E6-AP on the normal breast development and breast tumor development, we have acquired an E6-AP null mouse line. These models will be helpful in understanding the role of E6-AP in the development and progression of breast tumors. Presently, we are in the process of breeding and analyzing the mammary gland development of both the overexpression and the loss of E6-AP expression models. Our data from these models suggest that overexpression of E6-AP in mammary gland results in impaired mammary gland development. Furthermore, loss of E6-AP expression results in an overly developed mammary gland compare to that of controls mammary gland. These mice exhibit increased ductal branching and alveolar buds. In order to study the expression profile of E6-AP in human breast tumors, we examined 56 advanced stage human breast cancer biopsy samples. We found an inverse correlation between the expression of E6-AP and the expression of estrogen receptor-alpha in these tumors. The Spearman Rank Correlation Coefficient is 0.38 and the p value is 0.004, indicating that this correlation is statistically significant. These data suggest a possible role of E6-AP in mammary gland development and tumorigenesis.

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Introduction

Breast cancer is the leading cause of death in American women. It is anticipated that one woman out of ten will develop breast cancer at some point during her life (20, 38, 43-45, 49). Although in recent years significant progress has been made in detection and treatment of the disease, much of the molecular basis of the disease remains unknown. This fact highlights the need to identify and understand the molecular basis associated with breast cancer development and progression.

Steroid hormones, estrogen and progesterone, play important role in the development and progression of breast cancer (3, 9, 10, 13). Estrogens and progesterones exert their biological effects on target tissues through intracellular receptor proteins, estrogen (ER) and progesterone (PR) receptors (16, 46, 63). These receptors contain common structural motifs which include a less well conserved amino-terminal activation function (AF-1) that effects transcription efficiency, which has the hormone-independent activation function; a central DNA-binding domain, which mediates receptor binding to specific DNA enhancer sequences and determine target gene specificity; and a carboxy-terminal hormone-binding domain (HBD). The HBD contains activation function-2 (AF-2); the region mediates the hormone-dependent activation function of the receptors (16, 46, 63).

In order to activate gene transcription, ER and PR undergo a series of well-defined steps. When bound to hormone, these receptors undergo a conformational change, dissociation from cellular chaperones, receptor dimerization, phosphorylation, interaction with coactivators and recruitment of chromatin modifying enzyme activities such as histone acetyl transferase activity (HAT) and ATPase activity, DNA-binding at an enhancer element of the target gene, and subsequent recruitment of basal transcription factors to form a stable preinitiation complex (PIC) (6, 21, 35, 36). These events are followed by up- or down-regulation of target gene expression.

Coactivators represent a growing class of proteins, which interact with receptors in a ligand-specific manner and serve to enhance their transcriptional activity. Prior to their identification, coactivators were predicted to exist based upon experiments, which showed that different receptors compete for a limiting pool of accessory factors required for optimal transcription. Stimulation of one receptor resulted in trans-repression of another receptor, indicating the depletion of a common coactivator pool (4, 37, 57). A number of coactivators have been cloned to date, including SRC-1 (48), TIF2 (GRIP1) (18, 19, 65, 66), p/CIP (ACTR/RAC3/AIB1/TRAM-1) (2, 5, 31, 60, 62), PGCs (54), SRA (29), CBP (1, 15, 26) and **E6-associated protein (E6-AP)** (42) etc. and this list is growing rapidly day by day.

Coactivators were originally envisioned to serve a bridging role, linking the receptor to the basal transcription machinery (53, 61). Recently, the functional role of coactivators has expanded by the observation that they have been shown to possess enzymatic activities that may contribute to their ability to enhance receptor mediated transcription; SRC-1, p300/CBP, and ACTR (RAC3/AIB1) possess a histone acetyl transferase, HAT, activity (2, 6, 11, 31, 36, 47, 59) and members of SWI/SNF complex contain an ATPase activity (12, 39, 55, 67). Ligand-activated receptors are thought to bring HAT and ATPase activities containing coactivators to the chromatin surrounding the receptor, disrupting the local repressive chromatin structure by

acetylating histones and possibly other chromatin associated factors and catalyzing the uncoupling of ionic interactions between histones and their substrate DNA (12, 39, 47, 55, 59, 67, 70). Because of their ability to enhance receptor mediated gene expression, coactivators are thought to play an important role in regulating the magnitude of the biological responses to hormones (30, 35, 68, 69). The level of coactivator expression is critical in determining the activity of the receptor in target tissues and variations in hormone responsiveness seen in the population may be due to differences in coactivator levels.

It is accepted that coactivators either possess or bring HAT and ATPase activities to the promoter region of the target genes and presumably manifest part of their in vivo coactivation functions through these enzymatic activities (12, 39, 47, 55, 59, 67). Recent identification of the enzymes of the ubiquitin-proteasome and ubiquitin-like pathways as coactivators by my own laboratory and others added a new twist to the coactivator field. These studies suggest that the ubiquitin-conjugating enzymes (UBCs) and the E3 ubiquitin-protein ligases, E6-AP and RPF1/RSP5, interact with members of the steroid hormone receptor superfamily including ER and PR and modulate their transactivation functions (27, 36, 42, 51, 52). Similarly, another coactivator protein, yeast SUG1, an ATPase subunit of the 26S-proteasome complex also interacts with and modulates steroid hormone receptor function (14, 33, 34). Instead of HAT activity, this group of coactivators possesses other enzymatic activities such as ubiquitin conjugation, ubiquitin ligation and protease activities. However, a common theme between the two groups of coactivators is that both possess some sort of enzymatic activity.

As mentioned above, my laboratory has identified ubiquitin pathway enzymes as coactivators of the nuclear hormone receptor superfamily. We have cloned an E3 ubiquitin-protein ligase, E6-AP as steroid hormone receptor interacting protein using a yeast two-hybrid screening assay. E6-AP enhances the hormone-dependent transcriptional activity of steroid hormone receptors, PR, ER, androgen (AR) and glucocorticoid receptors (GR) (42). E6-AP was previously identified as a protein of 100 kDa, present both in the cytoplasm and the nucleus. E6-AP mediates the interaction of human papillomaviruses type 16 and 18 E6 proteins with p53, a growth-suppressive and tumor-suppressive protein. The E6/E6-AP complex specifically interacts with p53 and promotes the degradation of p53 via the ubiquitin-proteasome protein degradation pathway (24, 25). As mentioned above, E6-AP is a member of the E3 class of functionally related ubiquitin-protein ligases. E3 enzymes have been proposed to play a major role in defining substrate specificity of the ubiquitin system (22, 23, 56). Protein ubiquitination also involves two other classes of enzymes, namely the E1 ubiquitin activating enzyme (UBA) and E2 ubiquitin conjugating enzymes, UBCs. The UBA first activates ubiquitin in an ATP-dependent manner. The activated ubiquitin then forms a thioester bond between the carboxyl-terminal glycine residue of ubiquitin and a cysteine residue of the UBA. Next, ubiquitin is transferred from the E1 to one of the several E2s (UBCs), preserving the high-energy thioester bond. In some cases, ubiquitin is transferred directly from the E2 to the target protein through an isopeptide bond between the ε-amino group of lysine residues of the target protein and the carboxyl-terminus of ubiquitin. In other instances, the transfer of ubiquitin from UBCs to target proteins proceeds through an E3 ubiquitin-protein ligase intermediate such as E6-AP (7, 8). The carboxyl-terminal 350 amino acids (aa) of E6-AP contains a "*hect*" (homologous to the E6-AP carboxy terminus) domain, which is conserved among all E3 ubiquitin protein-ligases and E6-AP related proteins characterized to date. The extreme carboxyl-terminal 100 aa contains the

catalytic region of E6-AP, which transfers ubiquitin to the protein targeted for degradation (22, 23). We have shown that the ubiquitin-ligase activity of E6-AP is not required for the coactivation function of E6-AP. It has been shown that the conserved cysteine (C) 833 residue in E6-AP forms a thioester bond with ubiquitin and is necessary for the transfer of ubiquitin to the proteins targeted for ubiquitination. The mutation of C833 to alanine (A) or serine (S) has been shown to eliminate the ubiquitin-protein ligase activity of E6-AP (22, 23). In cotransfection studies, we showed that an E6-AP bearing a C-to-S mutation at the critical site was still able to coactivate steroid hormone receptors. Furthermore, our data also indicate that the catalytic function located within the *hect* domain of E6-AP is not necessary for the ability of E6-AP to interact with and coactivate steroid hormone receptor function, further confirming that the ubiquitin-ligase activity of E6-AP is not necessary for E6-AP to function as a coactivator (42). These findings indicate that E6-AP possesses two independent, separable functions, coactivation and ubiquitin-protein ligase activity (42).

It has been shown that altered expression of one nuclear receptor coactivator; AIB1 contributes to the development of hormone-dependent breast and ovarian cancer. Interaction of AIB1, SRC-1, TIF2, and p/CIP with CBP/ p300 is important for the coactivation function. Thus, overexpression or loss of expression of any of these coactivators could potentially perturb signal integration by CBP/ p300 and affect multiple transduction pathways (2). Recenly, it has also been shown that another steroid receptor coactivator, SRA is also elevated in breast tumors (41). Furthermore, recently, we have also shown that E6-AP is overexpressed 2.5-4.5 fold in 90-95% of tumors using a mouse mammary model of multistage tumorigenesis. E6-AP is overexpressed only in tumors but not in the intermediate steps of tumorigenesis (58).

The purpose of this research is to explore the possibility that the altered expression of E6-AP may contribute to the development of breast cancer. In the original proposal, we proposed to explore this by developing animal models for overexpression and loss of function of E6-AP. To relate the observations obtained from these animal models to the clinical setting, we also proposed to study the expression patterns of E6-AP in various human breast tumor biopsy samples. In this progress report, we report that we have successfully generated E6-AP overexpression model. In this model, we have overexpressed coactivator protein, E6-AP in the mammary epithelium of mouse by using the mouse mammary tumor virus (MMTV) promoter. In order to study the effect of loss of function of E6-AP on the normal mammary gland development and mammary gland tumor development, we have acquired an E6-AP null mouse line. These models will be helpful in understanding the role of E6-AP in the development and progression of breast tumors. Presently, we are in the process of breeding and analyzing the mammary gland development of both the overexpression and the loss of E6-AP expression models. Our data from these models suggest that overexpression of E6-AP in mammary gland results in impaired mammary gland development. Furthermore, loss of E6-AP expression results in an overly developed mammary gland compared to that of the control mammary gland. These mutant mice exhibit increased ductal branching and alveolar buds. In order to study the expression profile of E6-AP in human breast tumors, we also examined 56 advanced stage human breast cancer biopsy samples. We found an inverse correlation between the expression of E6-AP and the expression of estrogen receptor-alpha in these tumors. The Spearman Rank Correlation Coefficient is 0.38 and the p value is 0.004, indicating that this correlation is

statistically significant. These data suggest a possible role of E6-AP in mammary gland development and tumorigenesis.

Body

In this original proposal, we hypothesized that E6-AP is an important modulator of the steroid hormone receptor mediated signal transduction pathway, and in cell growth and cycle control that are functionally significant in the development of breast cancer. In order to test this hypothesis we propose following objectives:

- **Development and analysis of an animal model for the overexpression of E6-AP in the mammary gland.**
- **Analysis of an animal model for loss of E6-AP expression in the mammary gland.**
- **Expression analysis of endogenous E6-AP and p53 in human breast tumor biopsy samples.**

Development and analysis of an animal model for the overexpression of E6-AP in the mammary gland.

In order to test the effect of overexpression of E6-AP on the development of normal mammary gland and development of mammary tumors, we have successfully generated a new transgenic mouse model. This model overexpresses E6-AP protein in the mammary epithelium. In order to target the expression of E6-AP protein to the mammary epithelium we have utilized the MMTV promoter. Several other investigators have also successfully used this promoter to target expression of transgenes to the mammary gland (32, 40, 50, 64).

Task 1. Design and generation of transgenic vector

To overexpress E6-AP in mouse mammary gland, a transgenic expression vector was generated (Fig. 1A). This vector contains the MMTV promoter fused to the human E6-AP cDNA. To enhance the expression of the transgene, the rabbit beta-globin gene fragment containing exon II, intron II, exon III and poly A signal sequences were also incorporated into the transgene vector. This vector contains the requisite splice acceptor and donor sites for maximum transgene expression. In order to distinguish transgene expression from endogenous mouse E6-AP, we fused flag tag to the amino-terminus of the E6-AP. In the original proposal, we proposed to use the Anti-Express tag but it turns out that Anti-Express tag is not sensitive enough to detect the expression of E6-AP using Western blot analysis. Therefore, we were forced to incorporated flag tag in the E6-AP transgenic expression vector instead of Anti-Express tag. Using flag tag we have successfully detected the expression of E6-AP by Western blot analysis (Fig. 1B).

The MMTV-flag-E6-AP transgenic expression vector was constructed as follows: initially a linker (5'-AATTCCCCGGG-3' and 5'-AATTCCCGGGG-3') containing the internal XmaI site was inserted into the E.CoRI site of the MMTVkBpA expression vector and resultant plasmid was named MMTVkBpAXmaI. To insert flag-tagged E6-AP into the MMTVkBpA expression plasmid, the full length E6-AP cDNA was amplified by polymerase chain reaction (PCR) with the primers containing flag tag sequences 5'-

TCCCCCCGGGATGGACTACAAGGACGACGATGACAAGGAAGCCTGCACGAATGAG-3' (upper strand) and 5'-TCCCCCCGGGTTACAGCATGCCAAATCCTTTGGCATACGTGATGGCCTT-3' (lower strand). The PCR product was digested with XmaI and cloned into the corresponding site of the MMTVkBpAXmaI. After sub cloning the PCR amplified cDNA of E6-AP was sequenced and was found to be correct in sequence and reading frame.

In order to determine whether the MMTV-flag-E6-AP expression vectors was able to express full length E6-AP protein, this vector was transiently transfected into HeLa cells and the expression of E6-AP was detected by Western blot analysis using anti-flag tag specific antibodies. As shown in Fig. 1B, MMTV-flag-E6-AP expression vector was able to express full length E6-AP protein compared to that of control vector, which does not contain flag-E6-AP cDNA. Furthermore, Fig. 1B also suggest that anti-flag antibody is able to detect E6-AP expression by Western blot.

Next we asked whether the flag-E6-AP protein functions as a coactivator of nuclear hormone receptors. Previously, we have shown that E6-AP acts as a coactivator of PR and ER in cells. To test the coactivation function of flag-E6-AP, the E6-AP expression plasmid along with receptor expression and reporter plasmids were cotransfected into HeLa cells. Then cells were treated with appropriate hormones and the activity of the reporter gene was measured. Fig. 2 suggests that in the absence of ligand, PR has a minimal effect on reporter gene expression either in the absence or in the presence of E6-AP. Addition of hormone increases the reporter gene activity in the absence of E6-AP; when flag-E6-AP was coexpressed with PR, the activity of PR was further stimulated by 4 to 5-fold. Similarly, the flag-E6-AP protein was also able to enhance the ER activity in HeLa cells (Fig. 3). These data suggest that flag-E6-AP is functional and it acts as a coactivator of PR and ER.

Task 2. Generation of transgenic animals

After establishing that the flag-tagged E6-AP protein is intact and biologically functional, the transgene was released from the transgenic expression vector by digesting MMTV-flag-E6-AP vector with NotI and KpnI enzymes. After purification from the vector backbone, the transgene DNA was extracted with phenol-chloroform and ethanol pracipitated. After precipitation, the transgene DNA was suspended in injection buffer and microinjected into fertilized FVB one-cell embryos. The injected embryos were then implanted into the oviducts of pseudopregnant recipient mothers. Once, animals were born, the transgenic founders were identified by PCR and/or Southern blot analysis.

Task 3. Identification of transgenic founders

In order to identify the transgenic lines, we have developed a PCR screening method. PCR screening is faster and cheaper compared to Southern blot screening. To develop PCR screen we designed 2 pairs of primer sets. The locations of these primers in the transgene are shown in Fig. 4. The primers 1 and 2 will generate a 385bp fragment and while the primers 3 and 4 will generate 450bp fragment (Fig. 4). The transgene-negative animals will not generate these bands. The sequence of the primers are as follows: primer 1, 5'-TGCTAACCATGTTCATGCC-3';

primer 2, 5'-CTCAGAGCAGGAGTTGTTGGG-3'; primer 3, 5'-ATGGACTACAAGGACGACGATG-3' and primer 4 5'-CCGGAAGCTCTGTACC-3'. In order to confirm the PCR result we have also performed Southern blot analysis of transgenic lines (Fig. 5). The lines, which are positive for transgene by PCR method, are also positive by Southern blot analysis (Fig. 5). By using PCR and Southern blot screening methods, we identified two transgene positive founder lines. However, only one founder was able to transmit the transgene to the offsprings. After another round of injection, we have identified two more transgenic lines. In total we have three transgene lines which can transmit transgene to their offspring. Finally, founders were bred with wild-type FVB mice to generate female mice for further analyses.

Task 4 and 5. Breed founders and analysis of expression patterns of transgene

Next, we analyzed transgenic lines for the expression of transgene human E6-AP in the mouse mammary gland. In order to confirm whether the human E6-AP transgene is expressed in mouse mammary gland, we analyzed the mammary glands of 8 weeks old virgin female mice by immunohistochemistry using an anti-E6-AP specific antibody obtained from Dr. N. J. Maitland. As a control, we also analyzed the mammary glands of the age matched wild-type non-transgenic animals. In order to study the expression profile of transgene, the mammary glands from 8 weeks old wild-type non-transgenic and transgenic virgin female mice were microdissected, fixed in 10% formalin and processed for immunohistochemistry studies using an anti-E6-AP specific polyclonal antibody produced in rabbit. This antibody recognized both human E6-AP and endogenous mouse E6-AP. As shown in Fig. 6, the human E6-AP transgene is highly expressed in the transgenic line E106 (Fig. 6A) and moderately expressed in transgenic lines E95 (Fig. 6B) and E37 (data not shown). Furthermore, transgene is specifically targeted to the mammary epithelium. Fig. 6 also suggests that the expression of endogenous mouse E6-AP is very low in the mammary gland. The control sections incubated with normal serum showed no signal (data not shown). We have also analyzed the expression of transgene by using anti-flag antibody. This analysis also demonstrates that human E6-AP transgene is highly expressed in the transgenic line E106 and moderately expressed in transgenic lines E95 and E37 (data not shown). These results were also further confirmed by Western blot analysis (data not shown). Presently, we are in the process of analyzing the expression pattern of transgene in different developmental stages of the mammary gland.

To determine the tissue specificity of transgene expression, total cellular extracts were prepared from various transgenic tissues (brain, liver, mammary gland and heart) and the expression pattern of transgene was compared with that of non-transgenic tissues by Western blot analysis using anti-E6-AP antibody. As expected endogenous E6-AP is highly expressed in brain and liver (Fig. 7). The expression of E6-AP is moderate in heart. However, low expression of endogenous E6-AP was detected in mammary gland. Fig. 7 shows that transgene human E6-AP is selectively overexpressed in mammary gland. However, the transgenic line E37 also showed some expression of transgene in lung (data not shown). The expression profile of our transgene is in agreement with that of published expression profile of other MMTV-transgenes. Again, transgenic line E106 showed highest expression of transgene and transgenic line E95 showed moderate expression of transgene (Fig. 7).

Task 6. Morphological and histological analysis of transgenic mammary glands

In order to analyze for physiological perturbations that could be attributed to the overexpression of E6-AP, we performed whole-mount analysis of transgenic and age-matched wild-type non-transgenic mammary glands at different stages of development, (a) virgin; (b) pregnant; (c) lactation; and (d) involution stages. In newborn mice, the mammary gland is comprise of a few ducts and it undergoes extensive growth post-natally. During puberty, the elongation and arborization of the ducts progress gradually into the surrounding mammary fatpad under the influence of gonadal hormones and terminate at the limits of the fatpad. With each subsequent estrous cycle, the lateral ductal branches subdivide progressively and give rise to small alveolar buds. During pregnancy additional ductal branching occurs and extensive lobular-alveolar proliferation gradually results in the complete filling of the fat pad at parturition (17). The whole mount analyses of 8 weeks old mammary glands from human E6-AP transgenic lines (E95 and E106) and wild-type non-transgenic lines were performed. As shown in Fig. 8, overexpression of transgene human E6-AP results in impaired mammary gland development compared to wild-type non-transgenic litttermates. The transgenic mammary glands failed to invade the entire fat pad. In contrast, the wild-type gland was able to invade the entire fat pad. In this study we used lymph node as a reference point to evaluate ductal outgrowth. In each group at least six animals were analyzed.

Next, we ask whether overexpression of transgene human E6-AP has any effect on the pregnant mammary gland. Again, we performed whole mount analysis on 15 days pregnant mammary gland from transgenic and non-transgenic mice. As shown in Fig. 9, the overexpression of human E6-AP has no significant effect on the pregnant mammary gland. The phenotype of the pregnant transgenic and non-transgenic mammary glands is identical. Both glands have identical lobular-alveolar proliferation.

In order to study the effects of overexpression of transgene human E6-AP on the involution process of the mammary gland, we analyzed 15 days involuting mammary glands from both transgenic and non-transgenic mice. Fig. 10 suggests that like pregnant glands, the transgene have no significant effect on the involution process. The involution is identical in transgenic and non-transgenic glands.

Task 7. Analysis of p53 in transgenic mammary glands

The expression analysis of p53 in transgenic mammary glands is in progress.

Analysis of an animal model for loss of E6-AP expression in the mammary gland.

The second aim of this proposal is to test the effects of loss of steroid hormone receptor coactivator, E6-AP, on the normal development of mammary gland and mammary gland tumors. In order to study the effect of loss of function of E6-AP on the normal breast development and breast tumor development, we have acquired an E6-AP null mouse line in our laboratory. Dr. Aurther Beaudet at the Baylor College of Medicine generated this line (28).

Task 8. Screening and breeding of E6-AP null mutant mice

To screen for the E6-AP null animals, we have also developed a PCR screening method. In order to develop the PCR screening method, we have designed 3 primers, which can differentiate between wild-type and null E6-AP locus. The sequence of the primers are: primer 1, 5'-ACTTCTCAAGGTAAGCTGAGCTTGC-3'; primer 2, 5'-GCTCAAGGTTGTATGCCTTGGTGCT-3' and primer 3, 5'-TGCATCGCATTGTCTGAGTAGGTGTC-3'. By using these 3 primers, we have successfully amplified the 750 bp fragment of wild-type E6-AP allele and 350 bp fragment of E6-AP null allele, respectively. The wild-type animals have only band of 750 bp, whereas homozygous E6-AP null animals have only 350 bp amplified fragment and heterozygous animals contain both bands (Fig. 11).

Task 9. Morphological and histological analysis of E6-AP null mutant mammary glands

In order to study the consequences of the loss of E6-AP expression on mammary gland development, the whole mount analyses of 12 weeks old virgin mammary glands from E6-AP null and wild-type mice were performed. As shown in Fig. 12A and B, loss of E6-AP results in increased lobular-alveolar buds compare to wild-type normal mammary glands. The E6-AP null mammary glands are more developed and the degree of mammary gland development is similar to that of a 5-10 days pregnant wild-type mammary gland. Increased lobular-alveolar buds in E6-AP null mammary glands compare to that of wild-type mammary glands are clearly visible at higher magnification in Fig. 12B.

Next, we ask whether loss of E6-AP expression has any effect on pregnant mammary gland. Again, we performed whole mount analysis on 15 days pregnant mammary gland from E6-AP null and normal wild-type mice. As shown in Fig. 13, the loss of E6-AP expression has no significant effect on the pregnant mammary glands. The phenotype of the pregnant E6-AP null and wild-type mammary glands is identical. Both glands have identical lobular-alveolar proliferation.

In order to study the effects of loss of E6-AP expression on the involution process of the mammary gland, we analyzed 15 days involuting mammary glands from both E6-AP null and wild-type mice. Fig. 14A and B suggests that like pregnant glands, the loss of E6-AP expression have no significant effect on the involution process. The involution is identical in transgenic and non-transgenic glands.

Task 10. Analysis of p53 expression in E6-AP null mutant mammary glands

The expression analysis of p53 in E6-AP null mutant mammary glands is in progress.

Expression analysis of endogenous E6-AP and p53 in human breast tumor biopsy samples.

The third aim of the proposal is to test the expression of endogenous E6-AP and p53 in human breast tumor biopsies. To date we have examined expression levels of E6-AP and ER in 56 different breast tumors and expression of p53 in 20 different tumors.

Task 11. Expression analysis of endogenous E6-AP and p53

To study the expression profile of E6-AP in human breast tumors, to date we have examined 56 advanced stage human breast cancer biopsy samples by Western blot using E6-AP specific antibody. Fig. 15 shows the expression of E6-AP in 20 different tumor samples. Majority of the tumors expresses E6-AP. Presently, we are in the process of confirming these results by immunofluorescent method using an anti-E6-AP antibody. We will also compare the expression profile of E6-AP from tumors to that of normal mammary tissue. Since, E6-AP is an E3 ubiquitin-protein ligase enzyme and recently, we have shown that ER is degraded through the ubiquitin proteasome pathway. Therefore, we also analyzed the expression profile of ER in breast tumors (Fig. 15) and then compare it with that of E6-AP expression. We found an inverse correlation between the expression of E6-AP and the expression of ER in these tumors. The Spearman Rank Correlation Coefficient is 0.38 and the p value is 0.004, indicating that this correlation is statistically significant.

It has been demonstrated that E6-AP promotes the degradation of p53 via the ubiquitin degradation pathway. Furthermore, in the brain of E6-AP knockout animals, the protein levels of p53 accumulate compared to those of normal littermates. Therefore, we also analyzed the endogenous expression of p53 protein from breast tumor biopsies. As shown in Fig. 15, p53 expression was not detectable in most tumors except tumor number 7, 10, 13 and 15. Furthermore, there was no statistical correlation between the expression profile of E6-AP and p53. Presently, we are analyzing more tumor samples for p53 expression.

Statement of work accomplished/in progress

Task 1. Design and generation of transgenic vector. **Accomplished.**
Task 2. Generation of transgenic animals. **Accomplished.**
Task 3. Identification of transgenic founders. **Accomplished.**
Task 4. Breed founders to assess expression and expand positive lines. **Accomplished.**
Task 5. Analysis of expression patterns of transgene in different development stages of mammary glands. **In Progress.**
Task 6. Morphological and histological analysis of transgenic mammary glands. **In Progress.**
Task 7. Analysis of p53 expression in transgenic mammary glands. **In Progress.**
Task 8. Breeding of E6-AP null mutant mice. **Accomplished**
Task 9. Morphological and histological analysis of E6-AP null mutant mammary glands. **In Progress.**
Task 10. Analysis of p53 expression in E6-AP null mutant mammary glands. **In Progress.**
Task 11. Expression analysis of endogenous E6-AP and p53. **In Progress.**

Key Research Accomplishments

- E6-AP transgenic expression vector has been generated
- Expression analysis of E6-AP from the transgenic vector has been completed
- The biological activity of the flag-E6-AP has been analyzed
- E6-AP transgenic mouse lines have been generated

- PCR based screening method for identification of transgenic animals has been developed
- E6-AP transgenic founders have been identified by both PCR and Southern blot
- Expression analysis of transgene has been analyzed in mammary glands
- The tissue specificity of transgene expression has been analyzed
- Analysis of E6-AP transgenic mammary glands has been done in virgin, pregnant and involution stages
- E6-AP knockout animals have been acquired
- PCR based screening method for identification of E6-AP knockout animals has been developed
- Analysis of E6-AP null mammary glands has been done in virgin, pregnant and involution stages
- Expression analysis of E6-AP in 56 different tumors has been done
- Expression analysis of ER in 56 different tumors has been done
- Expression profile of E6-AP has been compared with that of ER expression
- Expression analysis of p53 in 20 different tumors has been done

Reportable Outcomes

The ongoing work described here is going to be presented as a poster and an abstract at the Annual Endocrine Society Meeting (June 2001), in Denver Colorado (see appendix 2).

Conclusions

We have successfully generated an E6-AP overexpression model. In order to study the effect of loss of function of E6-AP on the normal breast development and breast tumor development, we have acquired an E6-AP null mouse line. Presently, we are in the process of breeding and analyzing the mammary gland development of both the overexpression and the loss of E6-AP expression models. Our data from these models suggest that overexpression of E6-AP in mammary gland results in impaired mammary gland development. Furthermore, loss of E6-AP expression results in an overly developed mammary gland compare to that of controls mammary gland. These mice exhibit increased ductal branching and alveolar buds. In order to study the expression profile of E6-AP in human breast tumors, we have examined 56 advanced stage human breast cancer biopsy samples. We found an inverse correlation between the expression of E6-AP and the expression of ER in these tumors. The Spearman Rank Correlation Coefficient is 0.38 and the p value is 0.004, indicating that this correlation is statistically significant. These data suggest a possible role of E6-AP in mammary gland development and tumorigenesis.

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Appendices

1. Figures 1-15

2. Abstract

Appendix 1

Figures 1-15

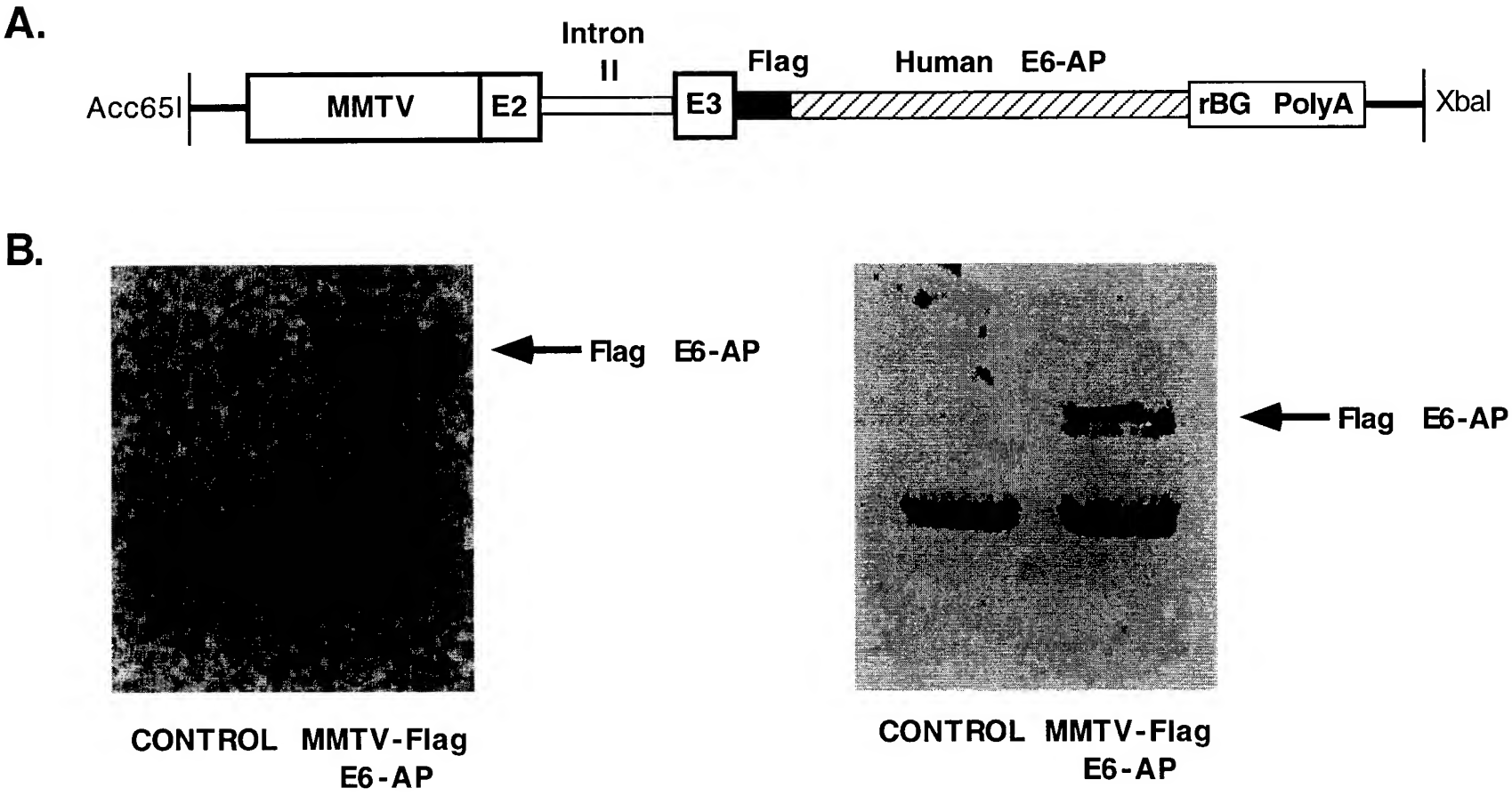


Figure 1: Generation and characterization of MMTV-E6-AP transgene. **A.** The MMTV-E6-AP construct contains the MMTV promoter and the full-length human E6-AP cDNA, fused to the exon II (E2), intron II, exon III (E3) and the rat beta-globin gene polyadenylation signal (rBG PolyA). **B.** HeLa cells were transiently transfected with either control plasmid or MMTV-Flag-E6-AP expression plasmid and the expression of E6-AP was detected by Western blot analysis using anti-flag tag specific antibody.

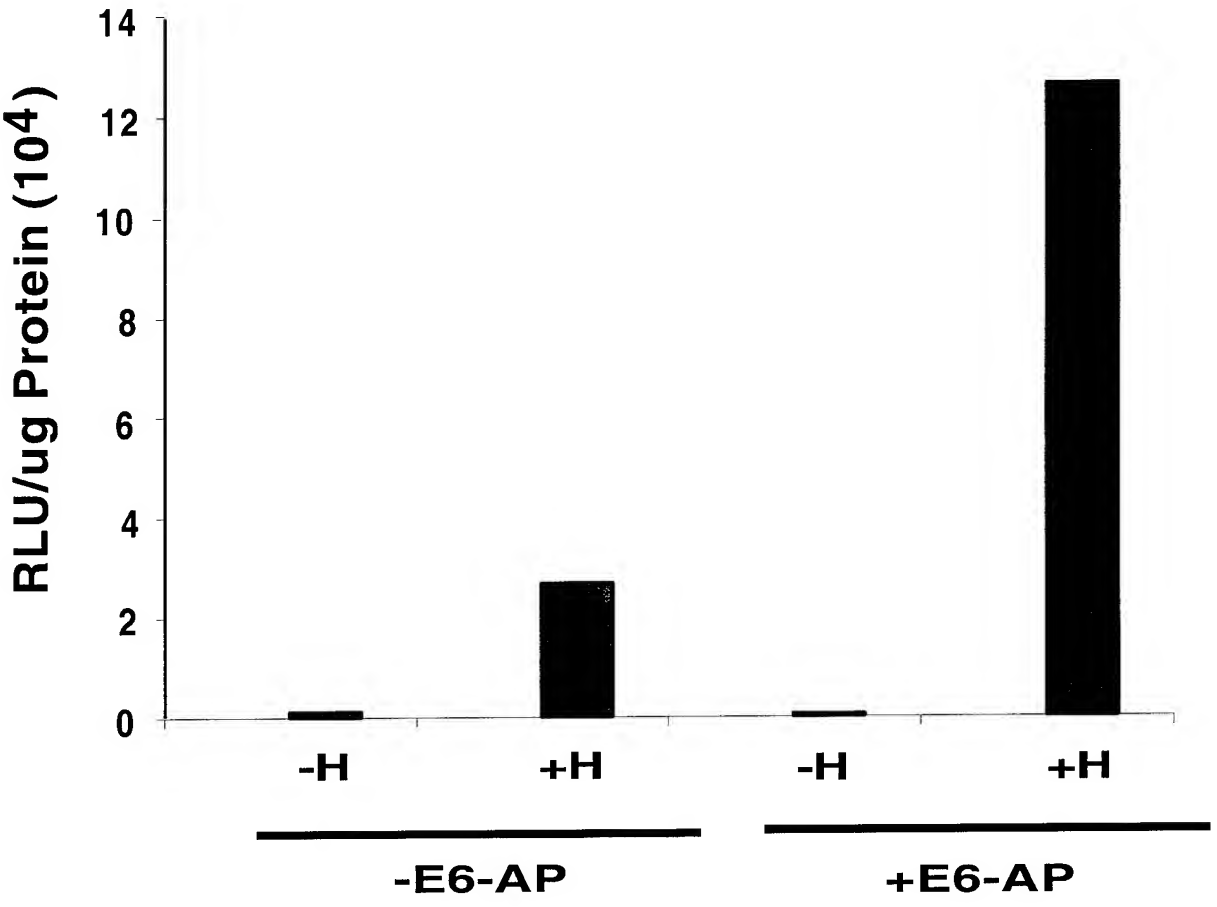


Figure 2: MMTV-Flag-E6-AP Coactivates PR Activity. Hela cells were transiently transfected with progesterone receptor expression plasmid and progesterone-responsive reporter plasmid in the absence or presence of E6-AP expression plasmid. The cells were treated with either vehicle (-H) or 10^{-7}M progesterone (+H). The data is presented as relative light units/ug protein (RLU/ug Protein).

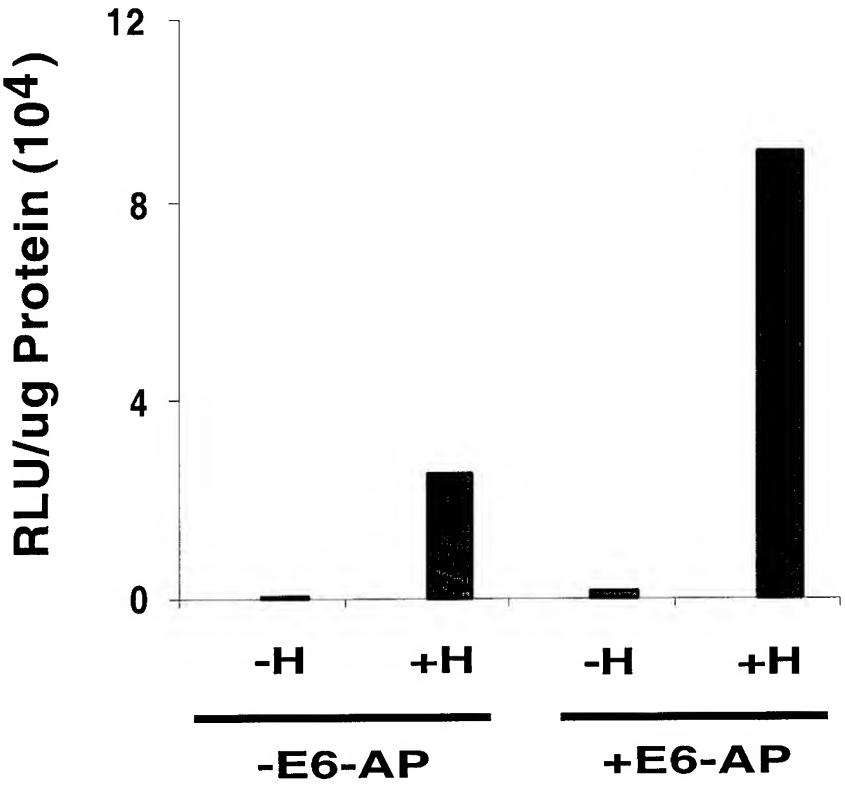


Figure 3: MMTV-Flag-E6-AP Coactivates ER Activity. Hela cells were transiently transfected with estrogen receptor expression plasmid and estrogen-responsive reporter plasmid in the absence or presence of E6-AP expression plasmid. The cells were treated with either vehicle (-H) or 10^{-7}M estradiol (+H). The data is presented as relative light units/ug protein (RLU/ug Protein).

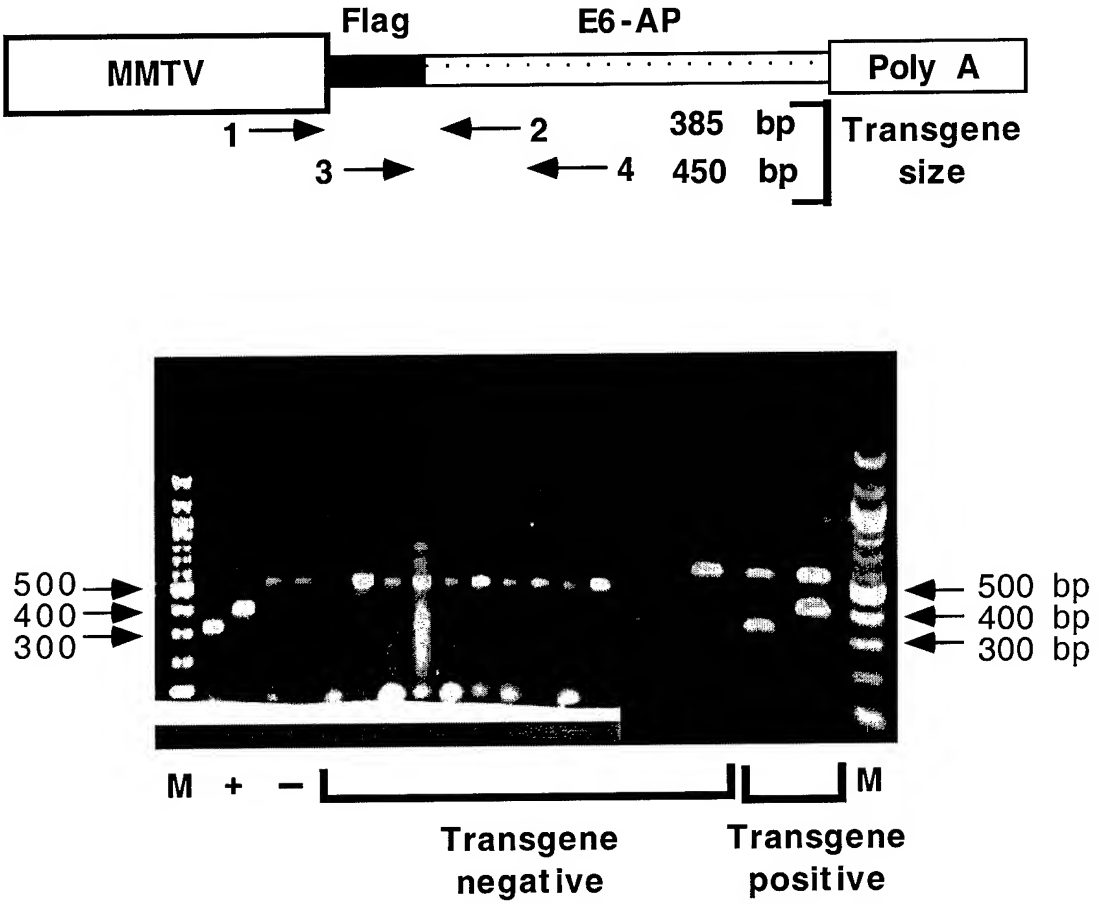


Figure 4: Screening of E6-AP transgenic lines by PCR method.
In order to identify the transgenic lines, a PCR screening method was developed . To develop PCR screen 2 pairs of primer sets were designed. The locations of these primers in transgene are shown in by arrow lines. The primers 1and 2 amplify a 385bp fragment and the primers 3 and 4 generate 450bp fragment. The transgene negative animals did not generate these bands.

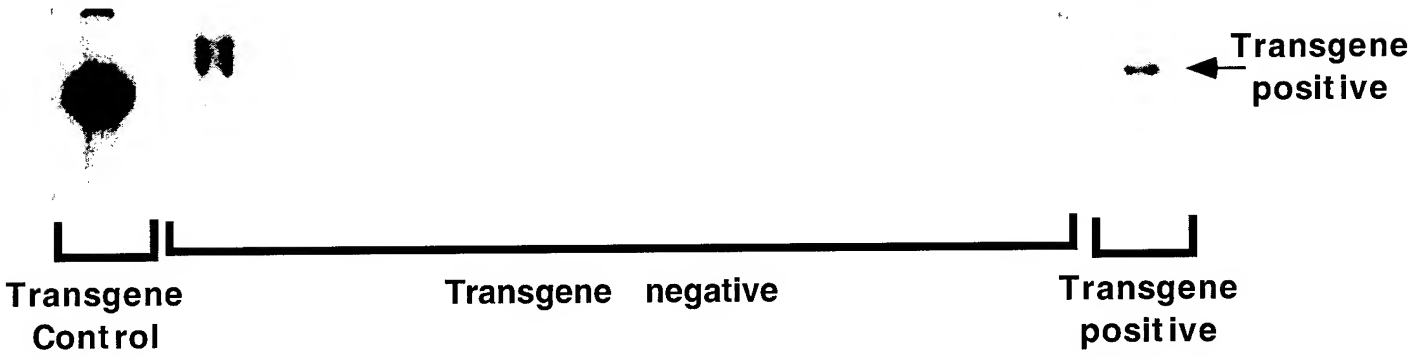
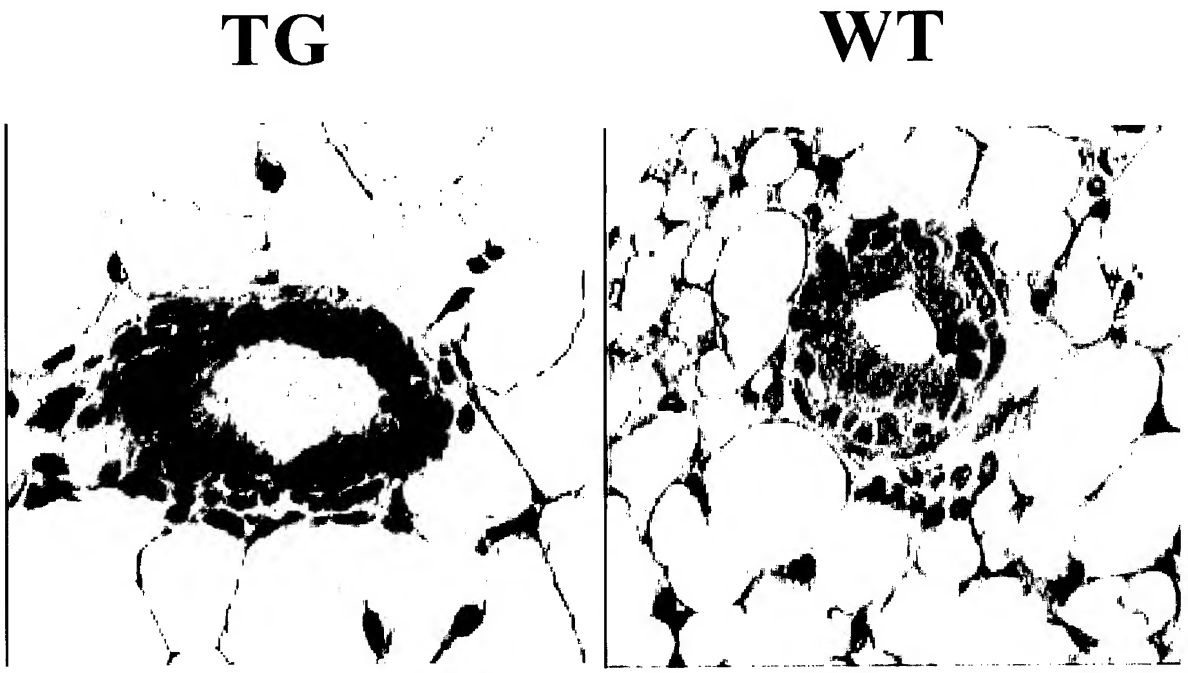


Figure 5: Screening of E6-AP transgenic lines by Southern blot.
Total genomic DNA was isolated from mice tails. The Southern blot was performed by using the 700 bp (BamHI-E.CoRI) long fragment of E6-AP as probe. The genomic DNA was digested with BamHI and BglII.

A.



B.

Figure 6: Expression analysis of transgene (MMTV-E6-AP) in the mouse mammary glands. E6-AP expression in the mouse mammary glands was analyzed by immunohistochemistry using an anti-E6-AP antibody. Positive signal is seen as dark (brown) spots. WT, Wild-type mammary gland; TG, E6-AP transgenic mammarygland. A. Transgenic line E106. B. Transgenic line E95.

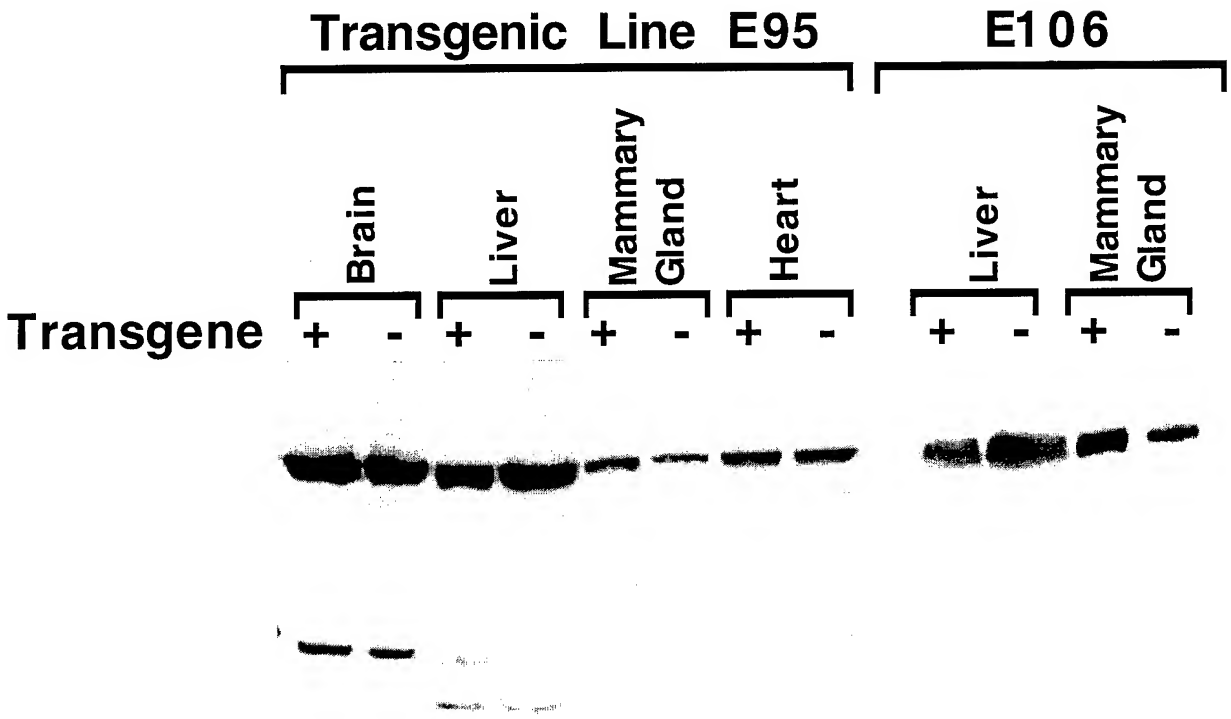


Figure 7: MMTV-driven E6-AP transgene preferentially overexpressed in mammary gland. Expression analysis of endogenous E6-AP and MMTV-driven human E6-AP transgene was performed in various mouse tissues such as brain, liver, mammary gland, heart etc. by Western blot using E6-AP specific antibody. MMTV-driven E6-AP transgene is specifically overexpressed in mammary gland.

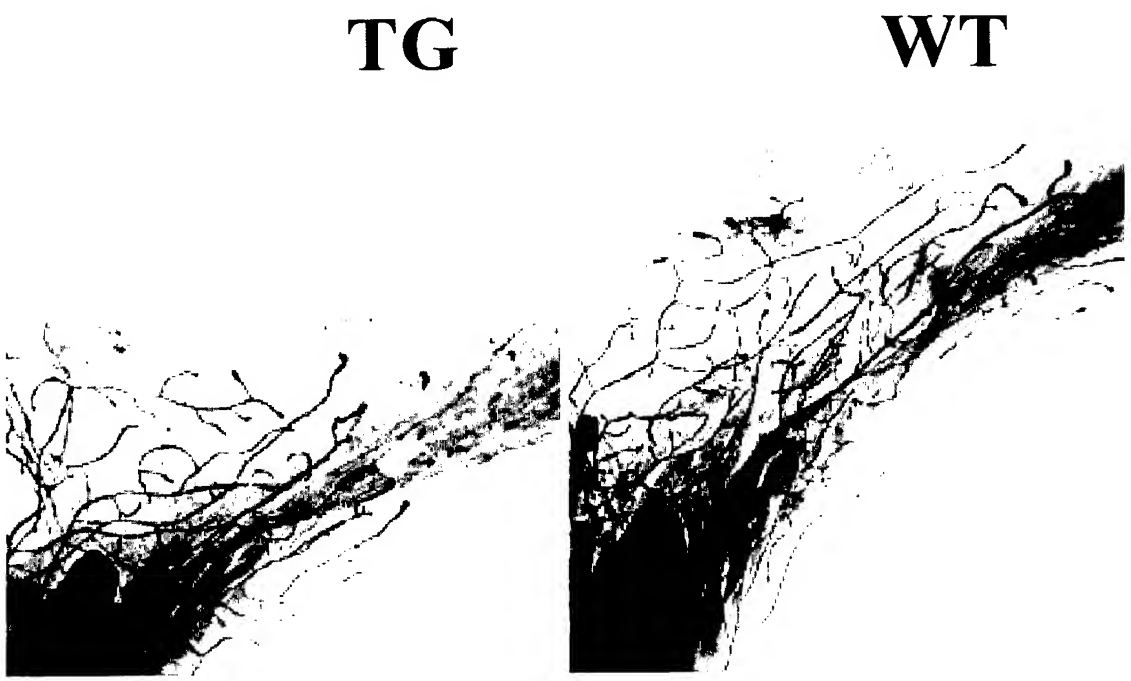


Figure 8: Overexpression of human E6-AP in mouse mammary gland results in impaired mammary gland development. Whole mount analyses of mammary glands from 8 weeks old virgin mice were performed from wild-type (WT) and MMTV-E6-AP transgenic mice (TG).

A.

B.

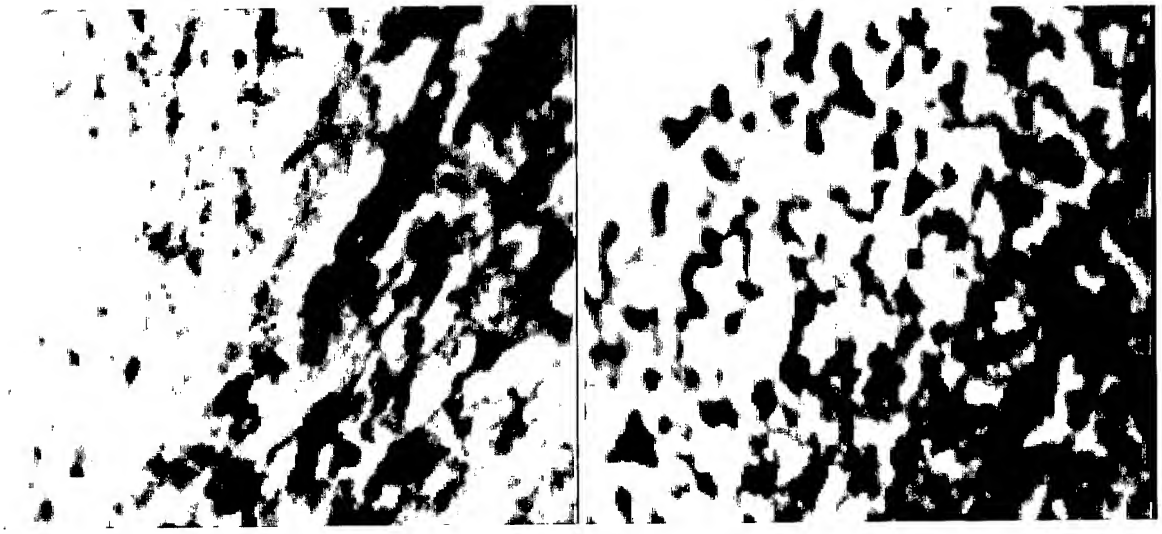
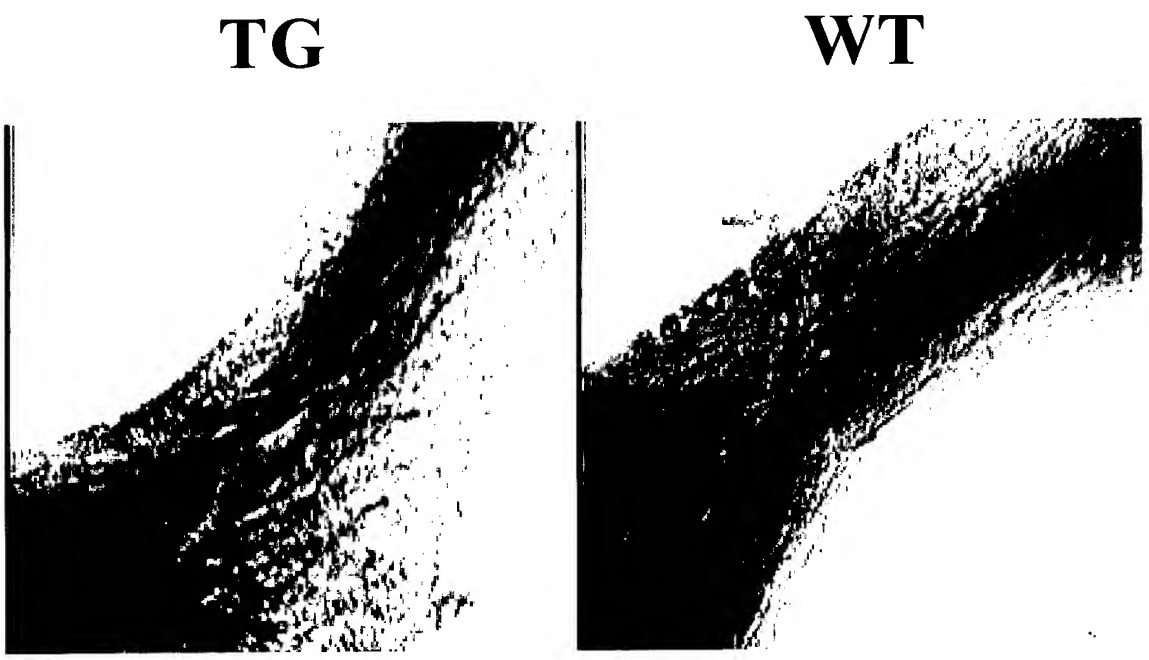


Figure 9: Overexpression of human E6-AP in mouse mammary gland has no significant effect on pregnant mammary glands. Whole mount analyses of mammary glands from 15 days pregnant mammary glands were performed from wild-type (WT) and MMTV-E6-AP transgenic mice (TG). (A) 5X (B) 20X

A.



B.

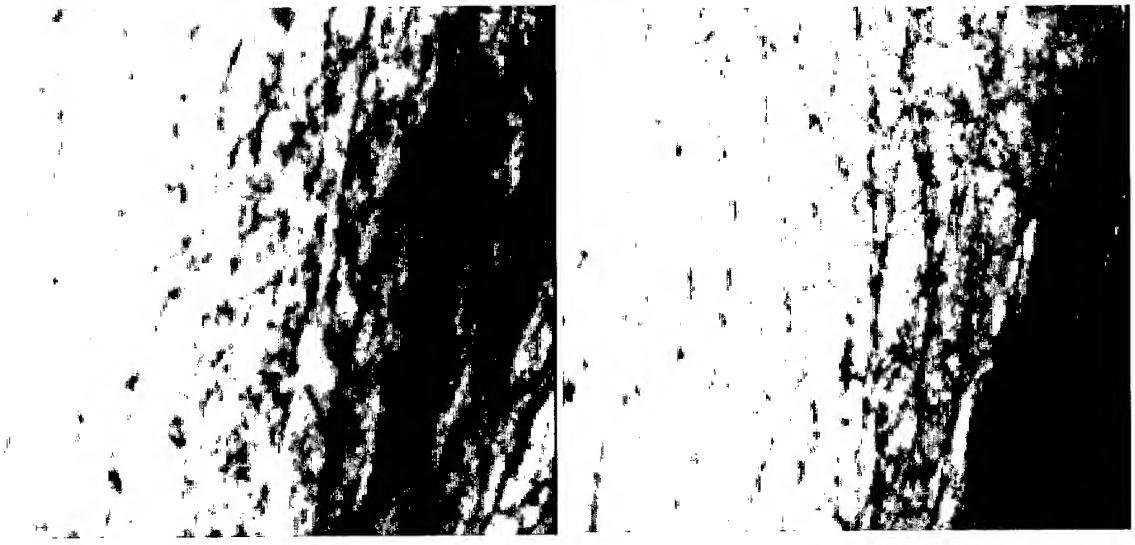


Figure 10: Overexpression of human E6-AP in mouse mammary gland has no significant effect on involuting mammary glands. Whole mount analyses of mammary glands from 15 days involuting mammary glands were performed from wild-type (WT) and MMTV-E6-AP transgenic mice (TG). (A) 5X (B) 20X

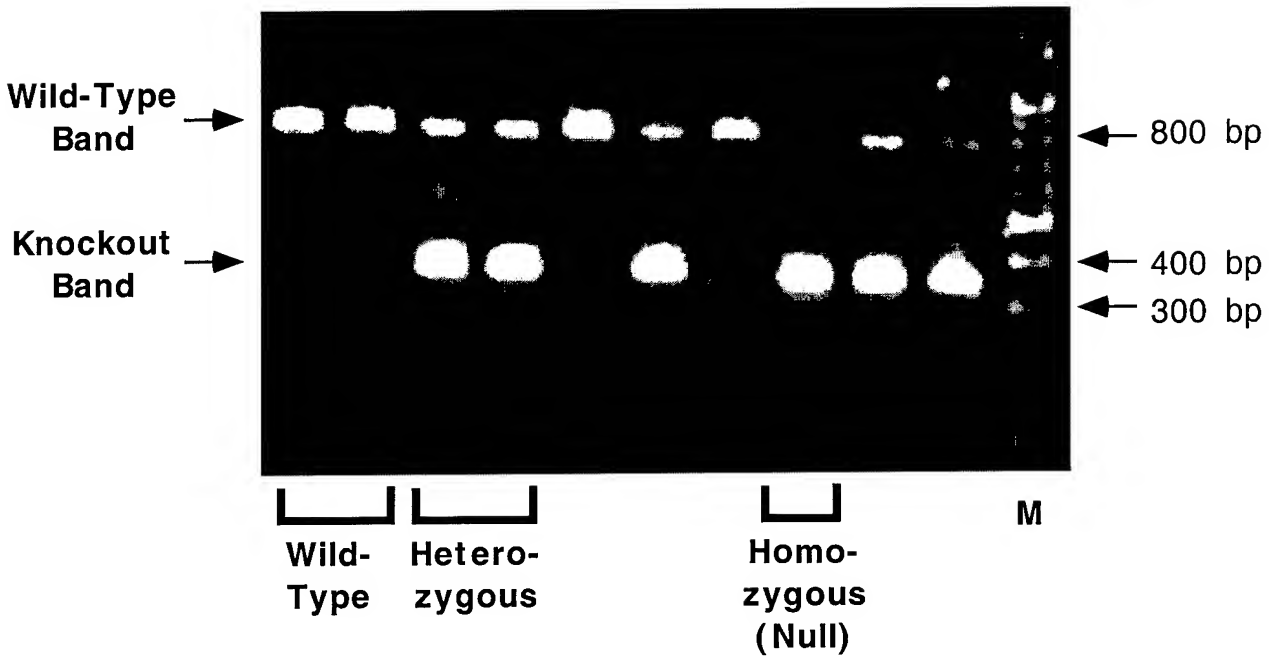


Figure 11: Screening of E6-AP KO (null) animals by PCR method.
In order to identify E6-AP null mice, a PCR screening method was developed . To develop PCR screen 3 pairs of primer sets were designed. These primers, successfully amplify the 750 bp fragment of wild-type E6-AP allele and 350 bp fragment of E6-AP null allele, respectively. The wild-type only generate a 750 bp long band, whereas homozygous E6-AP null animals generate only 350 bp long fragment and heterozygous animals contain both bands.

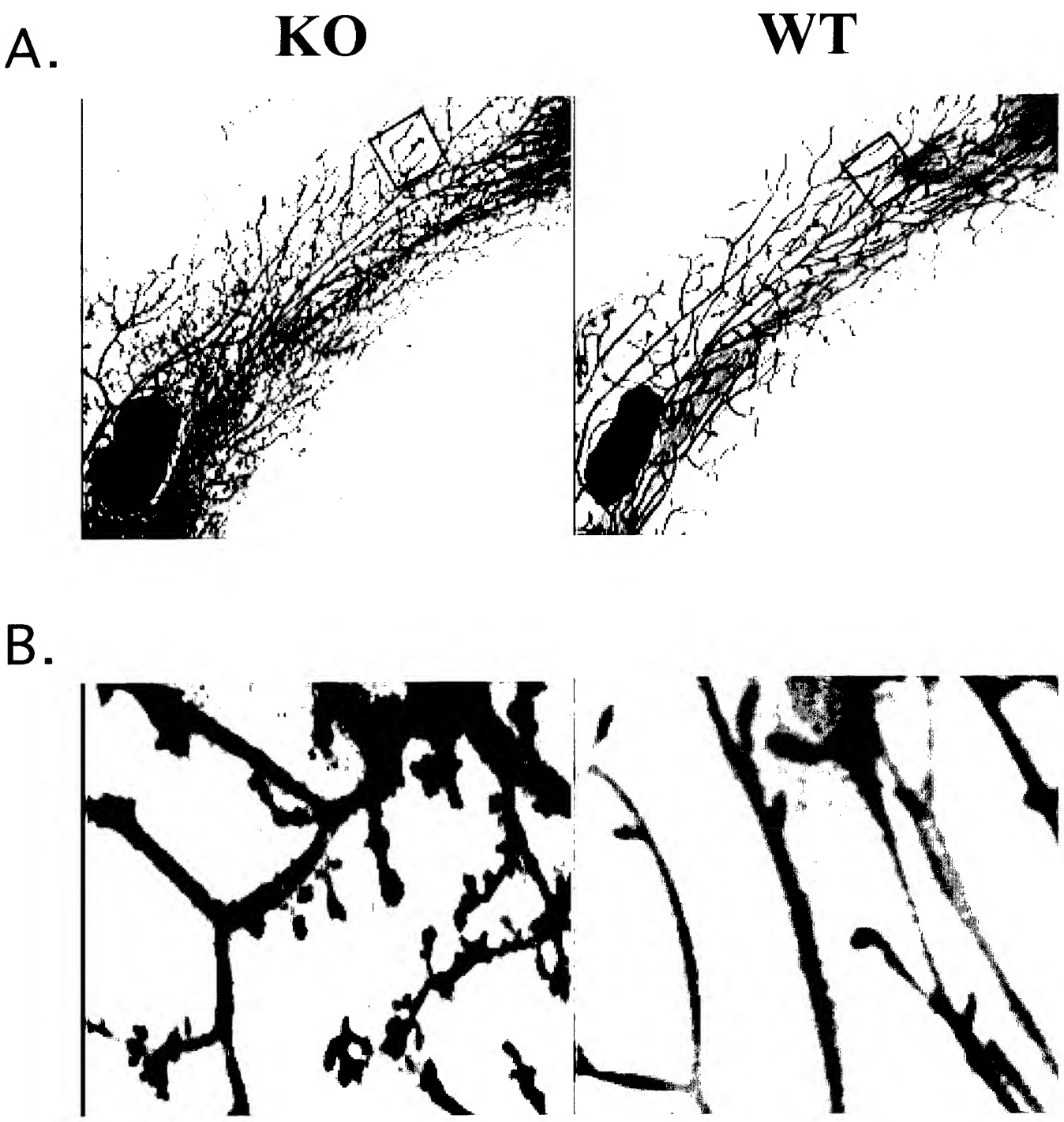


Figure 12: Loss of E6-AP expression results in increased alveolar buds. Whole mount analyses of mammary glands from 12 weeks old virgin mice were performed from wild-type (WT) and E6-AP knockout mice (KO). (A) 5X (B) 20X

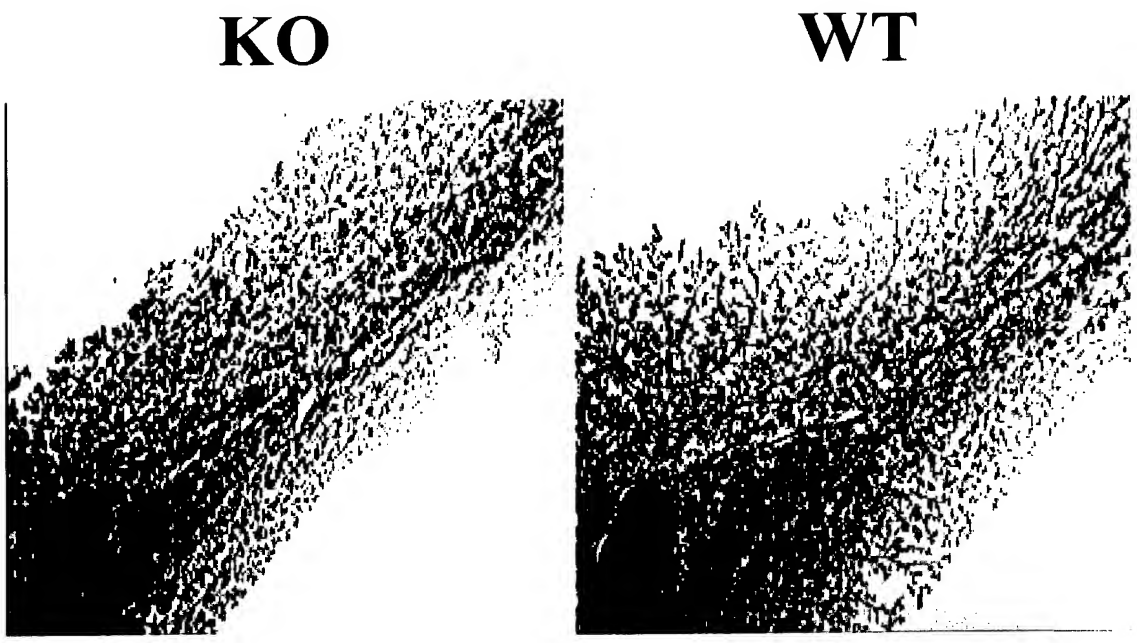


Figure 13: Loss of E6-AP expression has no significant effect on pregnant mammary glands. Whole mount analyses of mammary glands from 15 days pregnant mammary glands were performed from wild-type (WT) and E6-AP knockout mice (KO).

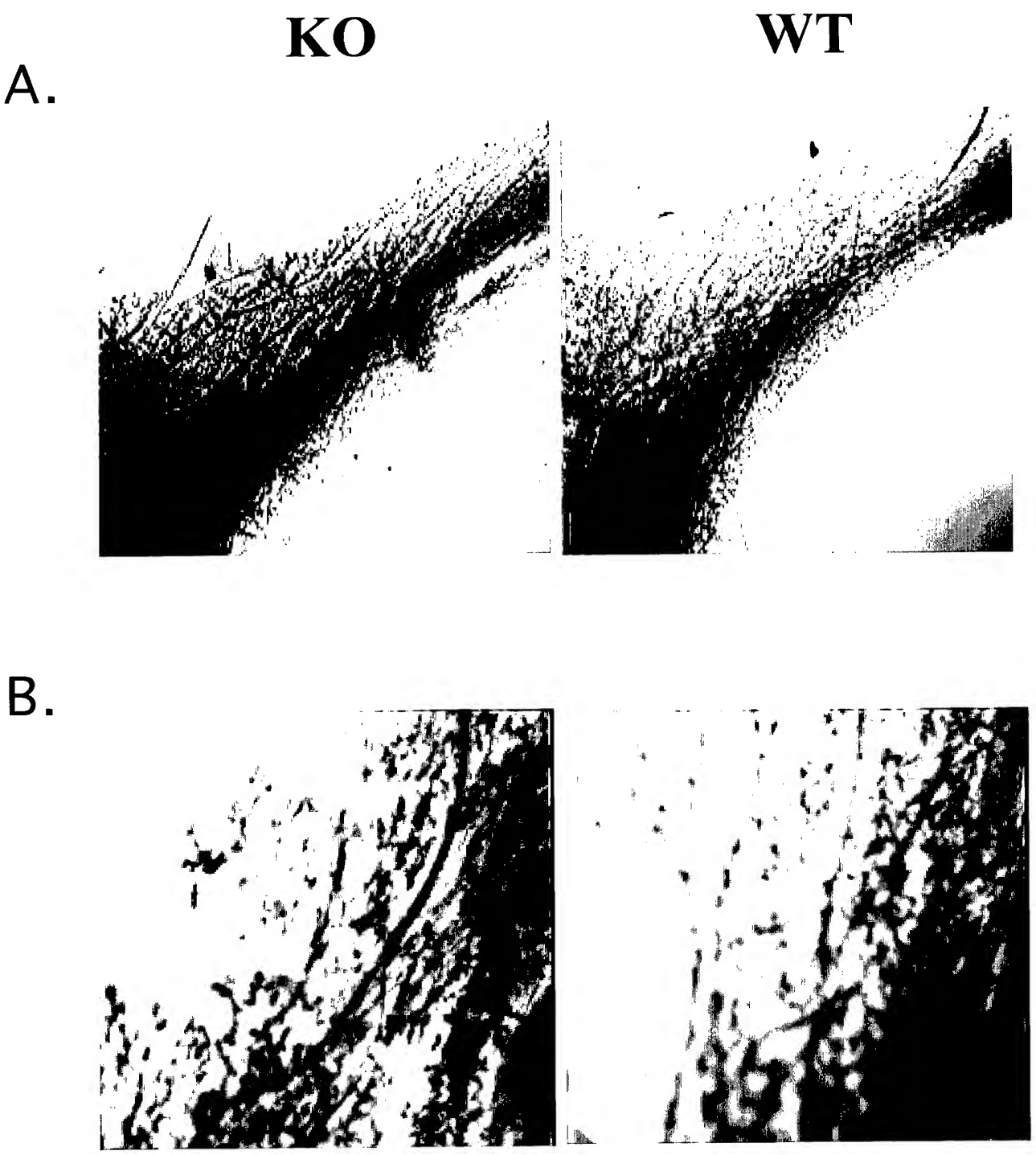


Figure 14: Loss of E6-AP expression has no significant effect on involuting mammary glands. Whole mount analyses of mammary glands from 15 days involuting mammary glands were performed from wild-type (WT) and E6-AP knockout mice (KO).(A) 5X (B) 20X

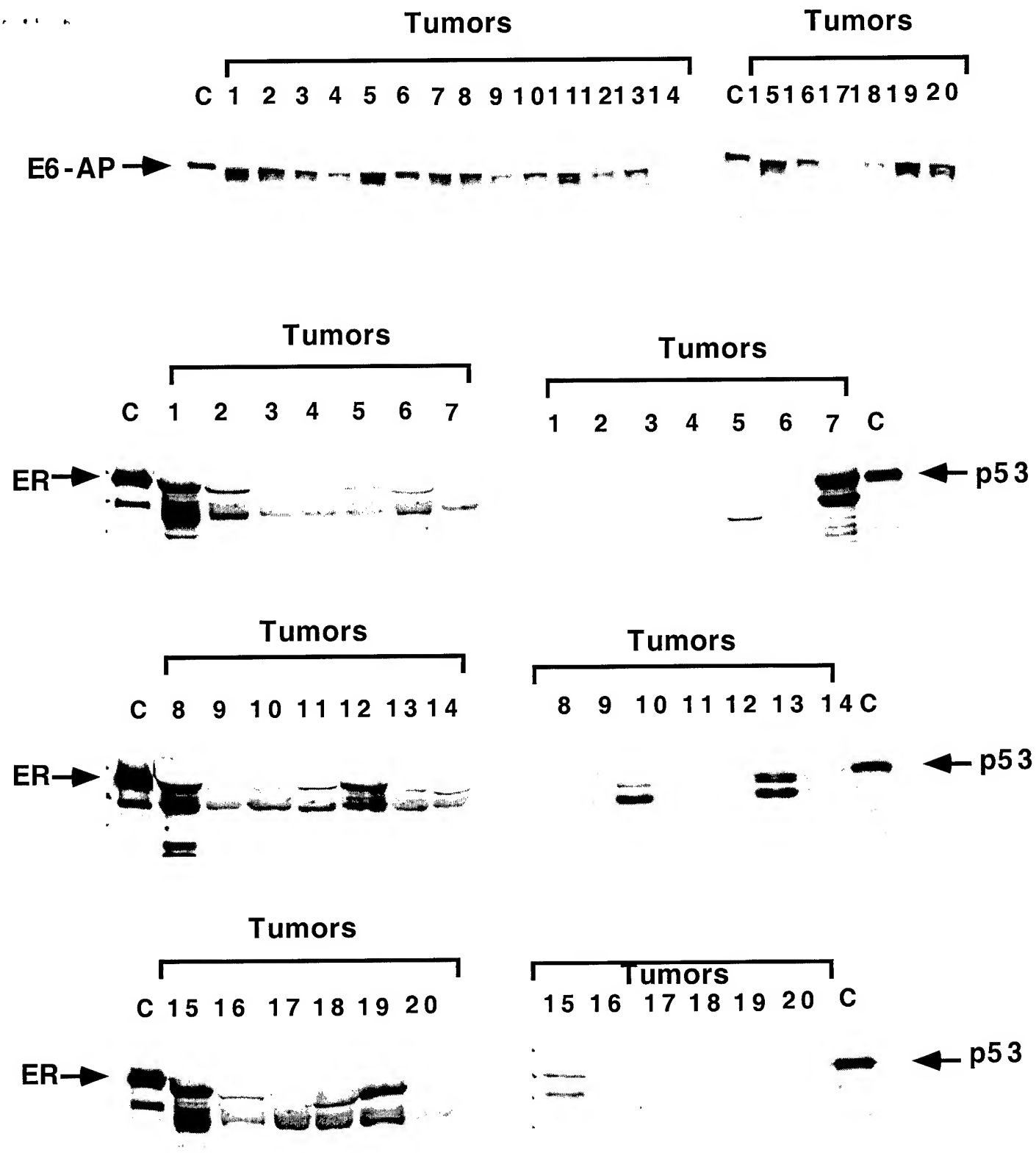


Figure 15: Expression analyses of E6-AP, ER and p53 in human biopsy tumor samples. Tumor samples were collected from the breast cancer center at the Baylor college of Medicine, Houston and expression of E6-AP, ER and p53 were determined by Western blot using E6-AP, ER and p53 specific antibodies.
C, control purified proteins.

Appendix 2

Abstract

Submission Type: General Submission - Poster Session
Category: Basic Science :: Hormones & Cancer - 09
Applying For Award: Travel Award
Sponsor: Zafar Nawaz
Character Count: 1951 (Max: 2500)
Keywords: tumorigenesis, coactivator, steroid receptor

Created: 30 Jan 2001 11:20 am
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All of the elements shown in the Abstract Preview screen below (including spaces) affect the character count.

INVOLVEMENT OF STEROID HORMONE RECEPTOR COACTIVATORS, E6-AP AND UBCS, IN THE DEVELOPMENT OF BREAST TUMORS.

X. Gao[1], G.M. Clark[2], F. Yan[1] and Z. Nawaz[1]. [1]Molecular and Cellular Biology, Baylor College of Medicine, Houston., TX, United States, [2]Breast Center, Baylor College of Medicine, Houston, TX, United States

Steroid hormones, estrogen and progesterone, are known to play a major role in the development of breast tumors by functioning through their cognate intracellular receptors, estrogen receptor (ER), and progesterone receptor (PR), respectively. Coregulators of steroid hormone receptors are important mediators of steroid receptors' function. Changes in the expression of these coactivators may contribute to mammary gland tumorigenesis. Recently, our laboratory identified several ubiquitin pathway enzymes, such as E6-associated protein (E6-AP) and ubiquitin conjugating enzymes (UBCs), as coactivators of steroid hormone receptors. Seperately, it was reported that E6-AP was overexpressed in a spontaneous mouse model of mammary gland tumorigenesis. To study the expression profiles of E6-AP and UBCs in human breast tumors, we examined 56 advanced stage human breast cancer biopsy samples. We found a correlation between the expression of E6-AP and the expression of ER-alpha in these breast tumors using Western blot analysis. The Spearman Rank Correlation Coefficient was 0.38 and the p value was 0.004, indicating that this correlation was statistically significant. Furthermore, the expression of E6-AP also correlated with that of UbcH7 (p=0.002), although the latter did not correlate with the expression of ER-alpha (p=-0.16). Our data provide the first evidence of a relationship between steroid hormone receptors and their coactivators, E6-AP and UBCs, suggesting a possible role of these coactivators in mammary gland tumorigenesis. It also indicates that E6-AP may be a potential target for breast cancer therapy.